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Evaluation of Interlaboratory Proficiency Surveys of Bilirubin Determinations in Sera of Newborns[1])

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Summary: We evaluated the results of two surveys of neonatal bilirubin determinations in 66 Slovakian clinical chemistry laboratories. Identical control samples prepared as described by *Vink* (Clin. Chem. *33* (1987) 1817–1821) and commercially available control serum were used for both surveys. These control samples were analysed in the participating laboratories using various modifications of diazo techniques based on the *Grof-Jendrassik* principle, and direct two-wavelength spectrophotometry according to *Vink* (Clin. Chem. *34* (1988) 67–70). The interlaboratory precision and accuracy of these two different analytical principles were compared. For diazo techniques the CVs varied from ± 6.8% to ± 10.3%, while the CVs for direct spectrophotometry were between ± 5.3% and ± 6.4%. Better results were obtained by recalculating the raw data of the analyses from all laboratories, using the reported results for the analysis of an identical calibrant. Accuracy was evaluated as the percentage of acceptable results (within ± 7% of the reference values). About 60% of the results obtained with diazo methods were considered acceptable, compared with about 22% of those obtained by direct spectrophotometry. However, results recalculated on the basis of the identical calibrant showed greater accuracy; acceptable results then varied between 74% and 82% of the total for the diazo techniques and from 83% to 91% for direct spectrophotometry.

We also describe the effect of haemoglobin and the effect of using an identical type of spectrophotometer on the results of direct spectrophotometry.

Introduction

A recent publication by *Vink* (1) reopened the discussion about the direct spectrophotometric determination of total bilirubin in sera from newborns. His proposal of a simple one-dilution method has certain advantages over the diazo methods, especially for the determination of this analyte in sera from neonates. In a recent paper (2), we confirmed the very close correlation between the results of direct spectrophotometry and those of the candidate reference method of *Doumas* (3).

We conducted surveys of bilirubin analyses in sera of newborns in 66 Slovakian clinical chemistry laboratories. Bilirubin is estimated in the majority of these laboratories by various modifications of the diazo methods based on the *Grof-Jendrassik*'s principle. *Vink*'s direct two-wavelength spectrophotometry provides a new opportunity for a high degree of standardization of the bilirubin methods applied to the sera of neonates.

The aims of this study are:

1) Evaluation of the interlaboratory precision and accuracy of direct spectrophotometry according to *Vink*, (1) by comparing the data from direct spectrophotometry with those from diazo methods in two neonatal bilirubin surveys.

[1]) Part of this contribution was presented as a poster at the XIV International Congress of Clinical Chemistry 22–26 July 1990 in San Francisco, California, USA

2) Investigation of the influence of an identical calibrant on the interlaboratory precision and accuracy of the two compared analytical principles under routine conditions in the participating laboratories.

3) Reevaluation of the effect of haemoglobin on the interlaboratory precision of the compared analytical principles.

4) Investigation of the effect of using the same type of spectrophotometer on the interlaboratory comparability of results of direct spectrophotometry.

Materials and Methods

Chemicals and reagents

1. Bovine serum albumin lyophilysed (ÚSOL manufacturer, Praha, Czechoslovakia)
2. Bilirubin cryst., anal. grade, product No. 15029 (Feinbiochemica SERVA, Heidelberg, Germany)
3. Caffeine reagent for the two-wavelength direct spectrophotometry was prepared as described by *Vink* (1). All chemicals used for the preparation were analytical grade and were supplied from LACHEMA corp., Brno, Czechoslovakia.

Control materials

Control samples designated "A", "B" and "C" were freeze-dried materials bottled in brown vials. Samples "A" and "B" were prepared as proposed by *Vink* (4) in bovine serum albumin, 40 g/l, and they had approximately the same content of bilirubin. Sample "B" also contained haemoglobin, corresponding to a concentration of about 1.3 g/l after reconstitution with distilled water.

Sample "C" was a freeze-dried control serum. Precibil, supplied by Boehringer Mannheim for use in the quality assurance of bilirubin determinations in sera of newborns.

Because samples "A" and "B" were not regular products of a manufacturer, the between-bottle variation of the bilirubin concentration was examined and compared with that of sample "C". Twenty vials of each control sample were reconstituted with an equal volume of distilled water and analysed on the BM/HITACHI 717 analyser. The variation of bilirubin content in control sera is shown in table 1.

Tab. 1. Between-bottle variation of bilirubin content in the samples after reconstitution with distilled water

	Sample A	Sample B	Sample C
$\bar{x}$ (μmol/l)	231.0	220.8	300.5
SD	0.97	0.96	1.17
CV (%)	0.42	0.43	0.39
n	20	20	20

All analyses were made in triplicate on a Boehringer Mannheim/HITACHI 717 analyser.

Methods

Two types of bilirubin assay were surveyed. One type is represented by the various modifications of *Grof-Jendrassik*'s principle which were actually in use in the participating laboratories. The other type is the direct two-wavelength spectrophotometry of *Vink* (1).

Calculations

Bilirubin concentration was determined by comparison with standards, or by the use of a calibration curve in case of the diazo methods. In *Vink*'s spectrophotometric method, the molar absorptivities at 465 and 528 nm were used as published for the calculation of the bilirubin concentration. The following formula was used:

$$c_{bil} = f \cdot \frac{A_{465} - A_{528}}{48000 - 970} \cdot 10^6 \text{ μmol/l},$$

where f is the reciprocal volume fraction of serum.

Organization of the quality control

We performed two surveys in 66 Slovakian laboratories, in which neonatal bilirubin is routinely estimated. Identical control materials, caffeine reagent, instructions and forms for the results were sent to all participants. In both surveys, identical lots of samples "A" and "B" were used, but different lots of sample "C".

The participants provided the results of their measurements and all the raw data of their analyses for the organizing centre. We divided the results into two groups according to methods used. For each of these groups we compared the "measured" and "recalculated" results. The "measured results" are the initial ones calculated and obtained in the participating laboratories. "Recalculated results" were obtained in the evaluating centre from the raw data, using sample "A" as an identical calibrant whose bilirubin content was estimated by the candidate reference method (3).

Results

Interlaboratory precision and accuracy

The results of interlaboratory precision are obvious from figure 1 and table 2. Interlaboratory CVs are remarkably dependent in all analysed samples on the methods used. We obtained CVs between ± 6.8% and ± 10.3% for the diazo methods. However, direct spectrophotometry showed better interlaboratory precision, with CVs ranging from ± 5.3% to ± 6.4%. After recalculation of the "measured results" using sample "A" as an identical calibrant, we obtained CVs between ± 5.4% and ± 6.2% for the diazo methods and from ± 4.5% to ± 5.8% for direct spectrophotometry.

Accuracy of results was evaluated by comparison with the values obtained by the candidate reference method of *Doumas* (3). Because the method of two-wavelength direct spectrophotometry gives results identical to those of the candidate reference method (2), we evaluated both groups of survey results against the same reference values. The reference values for samples "A" and "B", determined in three Slovakian regional reference laboratories, were "A" = 229 μmol/l and "B" = 218.5 μmol/l. For sample "C" the target values of the manufacturer were used. Samples "C" contained 300 μmol/l and 306 μmol/l of bilirubin in survey 1

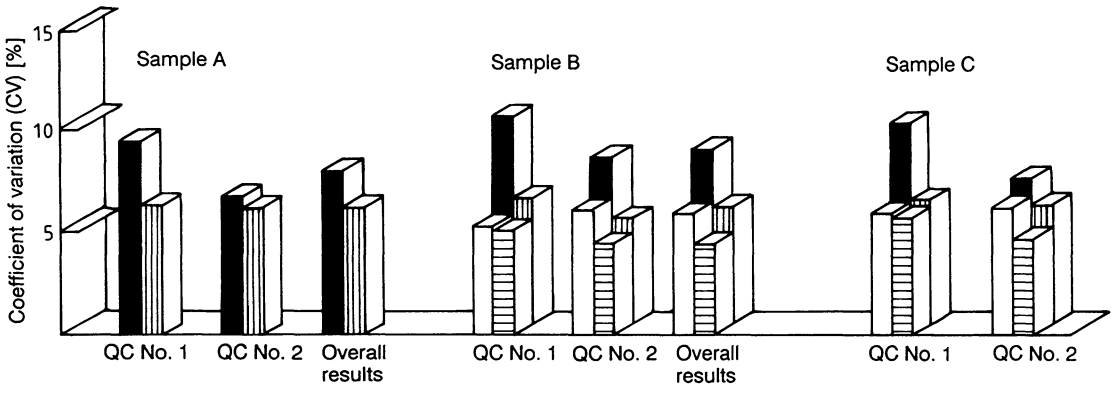

Fig. 1. Interlaboratory precision of results of quality control surveys.
Diazo techniques
□ recalculated results
■ measured results
Direct spectrophotometry
▤ recalculated results
▥ measured results

Tab. 2. Interlaboratory precision of results of two control surveys.

	Diazo techniques						Direct spectrophotometry					
	Sample A		Sample B		Sample C*		Sample A		Sample B		Sample C*	
	$\bar{x}$ (μmol/l)	CV (%)	$\bar{x}$ (μmol/l)	CV (%)	$\bar{x}$ (μmol/l)	CV (%)	$\bar{x}$ (μmol/l)	CV (%)	$\bar{x}$ (μmol/l)	CV (%)	$\bar{x}$ (μmol/l)	CV (%)
Survey 1	232.5	9.6	221.0 (217.8)	10.3 (5.4)	300.2 (295.4)	9.8 (5.9)	205.9	6.4	196.3 (218.6)	6.4 (5.3)	269.3 (300.2)	6.1 (5.8)
Survey 2	230.0	6.8	219.0 (218.9)	8.3 (6.2)	305.9 (302.2)	7.1 (6.2)	204.5	6.3	195.6 (219.5)	5.3 (4.5)	272.0 (304.6)	5.8 (4.6)
Overall results	230.7	8.0	218.9 (218.1)	8.7 (6.0)	– –	– –	205.1	6.3	195.9 (219.4)	5.9 (4.5)	– –	– –

Recalculated results using sample A as identical calibrant are listed in parentheses.
* No overall results were calculated because different lot numbers of PRECIBIL® were used in surveys 1 and 2.
Survey 1: 64 participants, held October 1989:
survey 2: 66 participants, held May 1990

and in survey 2, respectively. A limit of ± 7% of reference values which is proposed by NRL (National reference laboratory) was used for the evaluation. From table 3 it is obvious that the accuracy achieved is different in the two surveys, and depends on the methods used. The percentage of acceptable results varied from 13.0% to 31.7% for direct spectrophotometry and from 58.7% to 67.0% for the diazo methods. A high degree of accuracy was achieved in the group of "recalculated results", where acceptability varied from 73.9% to 81.8% for diazo methods from 83.3% to 91.5% for direct spectrophotometry.

Effect of haemoglobin on interlaboratory precision

As stated in Materials and Methods, samples designated "B" contained haemoglobin in a concentration of about 1.3 g/l. The effect of this haemoglobin on interlaboratory precision is obvious from figure 2. Interlaboratory precision of the diazo techniques is remarkably influenced by haemoglobin, as shown by the fact that the highest interlaboratory CVs were obtained for the analyses of sample "B". However, it seems that haemoglobin has no effect on interlaboratory CVs in direct spectrophotometry.

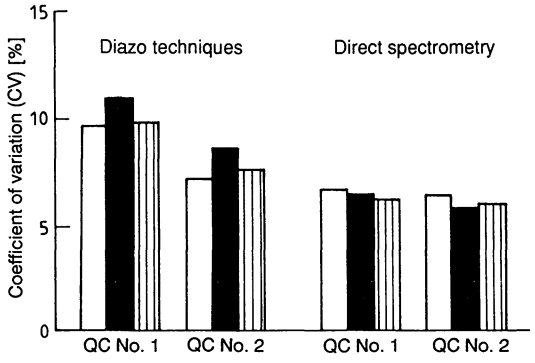

Fig. 2. Influence of haemoglobin on interlaboratory precision of results.
□ Sample A
■ Sample B
▥ Sample C

Tab. 3. Percentage of acceptable results (within + 7% of the reference values)

	Diazo techniques			Direct spectrophotometry		
	Sample A (%)	Sample B (%)	Sample C (%)	Sample A (%)	Sample B (%)	Sample C (%)
Survey 1	61.2	64.2 (81.8)	59.7 (79.1)	26.2	31.7 (83.6)	27.1 (87.1)
Survey 2	63.0	62.0 (73.9)	67.0 (78.0)	23.6	13.0 (91.5)	20.8 (83.3)
Overall results	62.0	63.3 (77.9)	63.3 (78.6)	25.0	22.8 (87.5)	24.1 (85.3)

Recalculated results using sample A as identical calibrant are listed in the parentheses.

Effect of using the same type of spectrophotometer

We evaluated the results for direct spectrophotometry in both surveys, using the data from 48 laboratories in which only the spectrophotometer SPEKOL 11 was used. The spectral bandwidth of this equipment is 11 nm. The mean values and appropriate CVs for the "measured" and "recalculated" results are summarized in table 4. From these data, it is clear that the lowest interlaboratory CVs can be achieved by appropriate standardization of direct spectrophotometry.

Tab. 4. Interlaboratory precision of direct spectrophotometry from 48 laboratories in which the spectrophotometer SPEKOL 11 was used.

	Sample B		Sample C	
	$\bar{x}$ (μmol/l)	CV (%)	$\bar{x}$ (μmol/l)	CV (%)
Survey 1	199.5 (219.2)	3.80 (4.16)	271.4 (297.0)	4.10 (4.22)
Survey 2	196.2 (219.8)	3.46 (4.47)	274.1 (305.2)	3.93 (4.55)

Recalculated results using sample A as identical calibrant are listed in parentheses.

Discussion

The results of our study show that dual wavelength direct spectrophotometry as proposed recently by *Vink* (1) provides better interlaboratory precision than the methods based on diazo techniques. Interlaboratory CVs are also lower for direct spectrophotometry after recalculation of results using an identical standard. Similar studies have been presented by other investigators (5–7). In the study of *Blijenberg* (5), the direct reading method with buffer dilution according to *Hertz* shows lower CVs for interlaboratory precision (± 4.2% to ± 5.1%) than the diazo techniques. In our study, the best results for interlaboratory precision were obtained for direct spectrophotometry, with CVs from ± 3.4% to ± 4.5% (see tab. 4).

The accuracy of survey results is dependent on the degree of standardization. From table 3, it is obvious that better results were achieved by diazo methods. We explain this by the fact that over 90% of participating laboratories used diazo techniques in routine investigations of bilirubin. These techniques are regularly monitored by internal and external quality control, and they are calibrated more or less identically. On the other hand, direct spectrophotometry is not used at present in most of these laboratories. The concentration of bilirubin was calculated using molar absorptivities published by *Vink* (1) according to formula 1 (see Material and Methods). We found that the results of direct spectrophotometry were shifted in comparison with those of the diazo methods. Considering that the majority of spectrophotometers used were SPEKOL 11 with a spectral band width of 11 nm, we explain the shift in the accuracy of results (see tabs. 2 and 3) by the differences in the correct wavelength setting. We made no prior check of the instruments (wavelength and spectrophotometer accuracy, etc.) because the participating laboratories were operating under "routine conditions".

However, "recalculated results" showed very satisfactory accuracy, with a significantly higher percentage of acceptable results. About 80% of results from diazo techniques are acceptable, while almost 90% of all results from direct spectrophotometry are acceptable, in accordance with the better interlaboratory precision of this method. Similar positive effects have been described by *Röhle* (7). The use of an identical standard for the calculation of bilirubin concentration is in accordance with the proposal of *Vink* (4).

We investigated the effect of haemoglobin on the interlaboratory precision using sample "B". The concentration of about 1.3 g/l was chosen, because *Vink* (1) and *Doumas* (3) reported that low concentrations did not interfere. Although we used a low haemoglobin concentration, remarkable differences can be seen between the interlaboratory CVs of samples "A", "B" and "C" for the diazo techniques. It is possible that the presence of this low level of haemoglobin in sample "B" exaggerates the effects of small deviations in the composition of the reaction mixture, which can occur in the reconstitution of the reagent in different laboratories. On the other hand, in direct spectrophotometry, the low amount of haemoglobin in the control sample can also amplify the effects of possible deviations in the correct wavelength setting, or the effects of different spectral properties of the spectrophotometers used.

The results of our study confirmed the reliability of *Vink*'s direct spectrophotometry, which depends on the degree of standardization, including uniform procedure, standard and appropriate quality control materials. In this connection, we consider that the values of molar absorptivities for bilirubin recently published by *Doumas* (8) represent an important contribution to improving the accuracy of bilirubin determinations.

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